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SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins)[1]

EFSA Panel on Contaminants in the Food Chain (CONTAM)[2, 3]

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ABSTRACT

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the consumption of spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) in shellfish. They are cyclic imines (CIs), a family of marine biotoxins. SPXs and GYMs are produced by the dinoflagellates *Alexandrium ostenfeldii* and *Karenia selliformis*, respectively. The organism producing PnTXs has not been identified but has been described as a peridinoid dinoflagellate. PtTXs are suggested to be bio-transformed from PnTXs in shellfish. No information has been reported linking CIs to poisoning events in humans. SPXs have been detected in Europe while GYMs have not been found. Recently PnTXs were identified for the first time in shellfish in Europe but PtTXs have not been detected. There are no regulatory limits for CIs in shellfish. The toxicological database for SPXs, GYMs, PnTXs and PtTXs is limited, comprising mostly acute toxicity studies. In view of the acute toxicity and the lack of chronic toxicity data for CIs, the CONTAM Panel considered that an acute reference dose should be established but due to the lack of data this was not possible. By comparing the lowest lethal dose (LD_{50}) values for SPXs (50 and 500 µg/kg body weight (b.w.) administered by gavage or in feed, respectively) and the estimated 95th percentile of exposure (0.06 µg/kg b.w.) a margin of exposure in the range of 1000-10000 was calculated. The mouse bioassay has traditionally been used to detect CIs. However, due to poor specificity and ethical concerns it is not considered an appropriate method. The receptor-based fluorescence polarisation method has been developed as alternative, but it needs further development. Liquid chromatography-tandem mass spectrometry methods would be of value for the quantification of CIs, but certified reference standards and reference materials are needed to allow method development and (inter-laboratory) validation.

KEY WORDS

Marine biotoxins, cyclic imines (CIs), spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs), pteriatoxins (PtTXs), shellfish, methods of analysis, human health, risk assessment.

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SUMMARY

Spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) are cyclic imines (CIs), and are a family of marine biotoxins present in shellfish. SPXs are produced by the dinoflagellate *Alexandrium ostenfeldii*, whereas GYMs are produced by the dinoflagellate *Karenia selliformis*. The organism producing PnTXs has not been identified yet but has been described as a peridinoid dinoflagellate. It has been suggested that PtTXs are bio-transformed from PnTXs in shellfish.

SPXs have been identified in a number of European countries bordering the Mediterranean Sea, Atlantic coast and the North Sea. To date GYMs have not been reported in shellfish produced in Europe, but they have been found in products imported from outside of Europe. Only recently PnTXs were identified for the first time in shellfish in Europe. They have so far only been identified in Norway. The other European countries have not conducted surveys to this end. The recent findings suggest that PtTXs are transformed from PnTXs in shellfish but PtTXs have not been reported in shellfish in Europe.

Although SPXs, GYMs, PnTXs and PtTXs are now known to occur in microalgae and/or shellfish in several parts of the world, no information has been reported linking these toxin groups to poisoning events in humans.

SPXs, GYMs, PnTXs and PtTXs, like other CIs such as prorocentrolides and spiro-prorocentrimines are macrocyclic compounds with imine (carbon-nitrogen double bond) and spiro-linked ether moieties. Due to similarities in chemical structure and toxicity in mice these different groups of toxins have been grouped together. SPXs are the largest group of the CIs. Presently 12 SPX-analogues are known of which 13-desmethyl SPX C is the most commonly found in shellfish. The chemical structures of three GYM-analogues have been characterised (GYM A-C). PnTXs are the CIs which are most closely related to the chemical structure of SPXs. Seven PnTX-analogues (PnTX A-G) have been chemically characterised. PtTXs and PnTXs are almost structurally identical and the latest results suggest that PnTX-analogues F and G are progenitors of all the known PnTXs and PtTXs via metabolic and hydrolytic transformation in shellfish. The chemical structures of PtTX A, B and C have been characterised.

Currently there are no regulatory limits for CIs in shellfish in Europe or in other regions of the world.

The toxicological database for CIs is limited and comprises only studies on their acute toxicity following intraperitoneal (*i.p.*) administration and oral (gavage, feed) administration. Based on the available information it can be concluded that CIs have the ability to bind and block acetylcholine receptors in the central- and peripheral nervous systems, including neuromuscular junctions.

There are no long term studies on CIs in experimental animals that would allow establishing a tolerable daily intake (TDI). In view of the acute toxicity of CIs the Panel on Contaminants in the Food Chain (CONTAM Panel) considered that an acute reference dose (ARfD) should be established for the different groups of CIs, but due to the lack of adequate quantitative data on acute oral toxicity (i.e. no-observed-adverse-effect levels (NOAELs)) this was not possible.

The CONTAM Panel calculated a margin of exposure (MOE) between the lowest lethal dose (LD_{50}) values for SPXs (in the region of 50 and 500 µg/kg body weight (b.w.) when administered by gavage or in feed, respectively) and the estimated 95th percentile of exposure (0.06 µg/kg b.w.) from consumption of shellfish currently on the market. The MOE is in the range of 1000-10000. The lower end of this range was based on the LD_{50} by gavage in fasted mice. The higher end of the range was based on the LD_{50} following administration of SPXs in the feed and is therefore more likely to be of relevance for the assessment of the risk of consumption of shellfish contaminated with SPXs. Taking also into account the steep dose response relationship observed in mice, and that mice given sublethal doses of SPXs are reported to make a rapid and full recovery, the CONTAM Panel concluded that

current estimated exposure to SPXs does not raise concern for the health of the consumer. The CONTAM Panel stresses, however, that this conclusion for SPXs is based on very limited toxicity data. Since exposure to other groups of CIs (GYMs, PnTXs and PtTXs) could not be estimated from the available data, no conclusions can be drawn with respect to any possible risk to consumers for these groups of CIs.

The mouse bioassay (MBA) has traditionally been used to detect CIs in shellfish. However, for reasons of animal welfare there is a growing concern with respect to its use. Due to its poor specificity it is not considered an appropriate detection method for CIs. The receptor-based fluorescence polarisation method has been shown to be able to detect GYM A and 13-desmethyl SPX C at relevant levels in shellfish but it needs further development. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific detection of individual CIs and they would be of value for their quantification in shellfish. None of the current methods of analysis to determine CIs in shellfish has been formally validated in interlaboratory studies. The CONTAM Panel noted that certified reference standards and reference materials for toxicologically relevant CIs need to be provided to allow method development and (inter-laboratory) validation.

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Marine biotoxins, also commonly known as shellfish toxins, are mainly produced by algae or phytoplankton.

Based on their chemical structure, the toxins have been classified into eight groups, namely, the azaspiracid (AZA), brevetoxin (BTX), cyclic imine (CI), domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX) and yessotoxin (YTX) groups, as agreed at the Joint FAO/IOC/WHO *ad hoc* Expert Consultation held in 2004[4]. Two additional groups, palytoxins (PlTX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhetic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysis toxins (DTX)), and AZA group toxins cause Azaspiracid Shellfish Poisoning (AZP). These toxins can all accumulate in the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems, and in extreme cases can lead to death.

To protect public health, monitoring programmes for marine biotoxins have been established in many countries, which often stipulate the use of animal models (for example, the mouse bioassay (MBA) and the rat bioassay (RBA)), for detecting the presence of marine biotoxins in shellfish tissues.

In the European Union (EU), bioassays are currently prescribed as the reference methods. Various stakeholders (regulators, animal welfare organisations, scientific organisations) have expressed their concerns about the current legislation in Europe, not only with regard to the use of large numbers of animals, involving procedures which cause significant pain and suffering even though non-animal based methods are available, but also since the scientific community argues that the animal test may not be suitable for all classes of toxins and that the state-of-the-art scientific methodology for the detection and determination of marine biotoxins is not fully reflected in current practices.

1. Legal framework

In 2004, the purported *EU Hygiene Package* of regulations, bringing together and replacing the existing hygiene regulations for the food sector previously contained in numerous individual vertical Directives was published. In Annex II Section VII Chapter V (2) to Regulation 853/2004/EC[5], are established maximum levels for ASP, PSP and DSP toxins. Annex III of Commission Regulation No 2074/2005/EC[6] of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to Regulation (EC) 854/2004[7], gives the monitoring authorities in the EU Member States the mandate to examine live molluscs for the presence of marine biotoxins. The *EU Hygiene Package* came into effect on 1 January 2006.

2. The Council Directive 86/609/EEC

Council Directive 86/609/EEC[8] makes provision for laws, regulations and administrative provisions for the protection of animals used for experimental and other scientific purposes. This includes the use of live vertebrate animals as part of testing strategies and programmes to detect identify and quantify

[4] ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf
[5] OJ L 139, 30.4.2004, pp. 55-205.
[6] OJ L 338, 22.12.2005, pp. 27-59.
[7] OJ L 139, 30.4.2004, pp. 206-320.
[8] OJ L 358, 18.12.1986, pp. 1-28.

marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food, and the avoidance of illness and disease.

Directive 86/609/EEC sets out the responsibilities that Member States must discharge. As a result of this use of prescriptive language, Member States have no discretion or flexibility, and most of the provisions of the Directive must be applied in all cases. It is clear that Member States have to ensure that: the number of animals used for experimental and other scientific purposes is reduced to the justifiable minimum; that such animals are adequately cared for; and that no unnecessary or avoidable pain, suffering, distress or lasting harm are caused in the course of such animal use.

Member States may not (Article 7, 2) permit the use of live animals in procedures that may cause pain, suffering, distress or lasting harm: "if another scientifically satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available". When animal use can be justified, Directive 86/609/EEC specifies a range of safeguards that Member States must put in place to avoid or minimise any animal suffering that may be caused. All justifiable animal use should be designed and performed to avoid unnecessary pain, suffering, distress and lasting harm (Article 8). Member States must ensure (Article 19, 1) that user establishments undertake experiments as effectively as possible, with the objective of obtaining consistent results, whilst minimising the number of animals and any suffering caused.

This latter requirement necessitates the use of minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane endpoints that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure. The EC and Member States are also required (Article 23, 1) to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, whilst providing the same level of scientific information.

3. Recognised testing methods for marine biotoxins and maximum levels

Commission Regulation (EC) No. 2074/2005[6] specifies a mouse bioassay (MBA) for the determination of paralytic shellfish poisoning toxins (PSP) and a MBA or the rat bioassay (RBA) for lipophilic marine biotoxins. Alternative test methods can be applied if they are validated following an internationally recognised protocol and provide an equivalent level of public health protection.

Besides paralytic shellfish poisoning toxins, okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids and yessotoxins, also cyclic imines, (gymnodimine, spirolides and others which are currently not regulated in the EU), all give a positive response in MBAs.

The reference method for the domoic acid group (the causative agent of ASP) is based on high-performance liquid chromatography (HPLC).

Chapter V (2) (c) and (e) of Section VII of Annex III to Regulation (EC) No 853/2004[5] establishes that food business operators must ensure that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

- 800 micrograms per kilogram for paralytic shellfish poison (PSP),
- 20 milligrams of domoic acid per kilogram for amnesic shellfish poison (ASP),

- 160 micrograms of okadaic acid equivalents[9] per kilogram for okadaic acid, dinophysistoxins and pectenotoxins in combination,
- 1 milligram of yessotoxin equivalents per kilogram for yessotoxins,
- 160 micrograms of azaspiracid equivalents per kilogram for azaspiracids.

4. Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004)

Based on the available information, the Joint FAO/IOC/WHO *ad hoc* Expert Consultation suggested provisional acute reference doses (ARfDs)[10] for the AZA, OA, STX, DA, and YTX-group toxins, respectively (summarized in the Table 1). The Expert Consultation considered that the database for the cyclic imines, brevetoxins and pectenotoxins was insufficient to establish provisional ARfDs for these three toxin groups. In addition, guidance levels were derived comparing results based on the consumption of 100 g, 250 g or 380 g shellfish meat by adults. However, the Expert Consultation noted that the standard portion of 100 g, which is occasionally used in risk assessment, is not adequate to assess an acute risk, whereas a portion of 250 g would cover 97.5 % of the consumers of most countries for which data were available.

Available methods of analysis were reviewed for the 8 toxin groups and recommendations made for choice of a reference method, management of analytical results and development of standards and reference materials.

The Joint FAO/IOC/WHO *ad hoc* Expert Consultation, however, did not have sufficient time to fully evaluate epidemiological data and to assess the effects of cooking or processing for deriving the provisional guidance levels/maximum levels for several toxin groups (especially the AZA and STX groups). The Consultation encouraged Member States to generate additional toxicological data in order to perform more accurate risk assessments and to facilitate validation of toxin detection methods in shellfish.

[9] Equivalents: the amount of toxins expressed as the amount of okadaic acid that gives the same toxic response followed intraperitoneal administration to mice. This applies similarly for the group of yessotoxins and azapiracids, respectively.

[10] The acute reference dose is the estimate of the amount of substance in food, normally expressed on a body-weight basis (mg/kg or µg/kg of body weight), that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of evaluation (JMPR, 2002).

Table 1: Summary data used in the derivation of the acute reference dose (ARfD) and current guidance levels.

Group toxin	LOAEL(1) NOAEL(2) µg/kg body weight	Safety Factor (Human data (H) Animal data (A))	Provisional ARfD[10]	Derived Guidance Level/ Maximum Level based on consumption of 100g (1), 250g (2) and 380g (3)	Limit Value currently implemented in EU legislation
AZA	0.4 (1)	10 (H)	0.04 µg/kg 2.4 µg/adult (a)	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
BTX			N/A		
Cyclic Imines			N/A		
DA	1,000 (1)	10 (H)	100 µg/kg 6 mg/adult (a)	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM (3)	20 mg/kg SM
OA	1 (1)	3 (H)	0.33 µg/kg 20 µg/adult (a)	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM
PTX			N/A		0.16 mg OA equivalents/kg SM
STX	2 (1)	3 (H)	0.7 µg/kg 42 µg/adult (a)	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM
YTX	5,000 (2)	100 (A)	50 µg/kg 3 mg/adult (a)	30 mg/kg SM (1) 12 mg/kg SM (2) 8 mg/kg SM (3)	1 mg/kg SM

SM: shellfish meat; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; N/A: not available; EU: European Union; OA – okadaic acid

(a): Person with 60 kg body weight (b.w.)

The Joint FAO/IOC/WHO *ad hoc* Expert Consultation also indicated that there were discrepancies between different risk assessments, especially for determining methods of analysis for certain marine biotoxins and in relation to established maximum limits.

Test methods for the eight toxin groups were reviewed and recommendations for Codex purposes made. Mouse bioassays are widely used for shellfish testing but for technical and ethical reasons it is highly desirable to move to new technologies which can meet Codex requirements more adequately. Most currently available methods do not meet fully the strict criteria for Codex type II[11] or III[12] methods and have therefore not been widely used in routine shellfish monitoring. However, the recommendations made by the Expert Consultation represent the best currently available methods. Liquid chromatography-mass spectrometry (LC-MS) has much potential for multi-toxin analysis and has been recommended for consideration and recommendation by Codex. The Joint FAO/IOC/WHO *ad hoc* Expert Consultation is of the opinion that the complexity and chemical diversity of some toxin

[11] A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.

[12] A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

groups is such that validated quantitative methods to measure all toxins within a group will be extremely difficult. Thus the implementation of a marker compound concept and the use of functional assays should be explored.

5. Working Group Meeting to Assess the Advice from the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Ottawa, Canada, April 10-12, 2006

The working group (WG) discussed available reference methods in particular and concluded that they should be highly specific, highly reproducible, and not prone to false positives or false negatives. The methods are expected to be definitive and may well result in significant rejections of products and must therefore withstand the most robust legal and scientific scrutiny.

In considering their weaknesses and merits, the meeting noted that the various mouse bioassays should be discussed individually since the level of performance and success differs markedly between the official method for PSP by mouse bioassay, the American Public Health Association (APHA) method for brevetoxins and the multiple mouse bioassay "DSP" procedures employed for the other lipophilic toxins such as okadaic acid, azaspiracids and others.

Recognizing that the majority of the currently available methods do not meet all Codex criteria for reference methods (Type II), the WG concluded that Codex Committee for Fish and Fishery Products (CCFFP) should consider a variety of biotoxin analytical methods. Wherever possible, reference methods should not be based on animal bioassays. Functional methods, biochemical/immunological and chemical-analytical methods currently in use, and considered to be validated according to Codex standards, should be recommended by CCFFP to the Codex Committee on Methods of Analysis and Sampling (CCMAS) for review and designation as Type II or Type III methods.

Because the Expert Consultation has offered 3 different guidance limits associated with three levels of consumption (100 g, 250 g and 380 g) for most toxin groups, it is important to determine which consumption level is appropriate for the protection of consumers.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002, the Commission asks EFSA to assess the current EU limits with regard to human health and methods of analysis for various marine biotoxins as established in the EU legislation, including new emerging toxins, in particular in the light of

- the report of the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004), including the ARfDs and guidance levels proposed by the Expert Consultation,
- the conclusions of the CCFFP working group held in Ottawa in April 2006,
- the publication of the report and recommendations of the joint European Centre for the Validation of Alternative Methods (ECVAM)/DG SANCO Workshop, January 2005,
- the report from CRL Working group on Toxicology in Cesenatico October 2005,
- any other scientific information of relevance for the assessment of the risk of marine biotoxins in shellfish for human health.

ASSESSMENT

1. Introduction

Spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) are cyclic imines (CIs), and are a family of marine biotoxins present in shellfish. They have only been discovered during the last few decades. The main producer of SPXs is the dinoflagellate *Alexandrium ostenfeldii*. This dinoflagellate has also been reported to produce saxitoxin (STX)-group toxins (Mackenzie et al., 1996; Cembella, 1998; Gribble et al., 2005; Kremp et al., 2009). SPXs were first isolated and characterised in scallops and mussels harvested in Nova Scotia, Canada in early 1990s (Hu et al., 1995). Due to environmental conditions, the profile of SPXs produced by *A. ostenfeldii* varies remarkably between the geographical locations (Cembella et al., 2000; Otero et al., 2010a).

Also a first GYM analogue (GYM A) was isolated and characterised in the beginning of 1990s in New Zealand oysters (Mackenzie et al., 1996; Seki et al., 1995, 1996) and later two additional GYM-analogues were isolated (Miles et al., 2000, 2003). Presently it is known that GYMs are produced by dinoflagellate *Karenia selliformis* (formerly *Gymnodinium selliforme*) (Seki et al., 1995; Miles et al., 2003; Haywood et al., 2004). MacKenzie et al. (2002) noticed that since GYM persisted in New Zealand oysters for several years after the initial contamination event, GYM A in shellfish might be a long term residue from the previous bloom of *K. selliformis*.

The organism producing PnTXs has not been identified yet but has been described as a peridinoid dinoflagellate (Rhodes et al., 2010). In 1995 a finding of the first PnTX-analogue (PnTX A) was reported in Japanese shellfish (Uemura et al., 1995). In the following year PnTX D was isolated from shellfish in Japan (Chou et al., 1996a). The isolation of PnTX-analogues B and C turned out to be more difficult due to their trace amounts in shellfish and therefore they were successfully isolated only in 2001 (Takada et al., 2001a). Recently new PnTX-analogues – E, F and G – were isolated from oysters in New Zealand and Australia (Selwood et al., 2010). The latest results suggest that PnTX-analogues F and G are produced separately in different dinoflagellates and are progenitors of all the known PnTXs and PtTXs via metabolic and hydrolytic transformation in shellfish (Selwood et al., 2010). Three analogues of PtTXs were isolated in shellfish in Japan in 2001 (Takada et al., 2001b), and it has been suggested that they are bio-transformed from PnTXs in shellfish (Selwood et al., 2010).

CIs were discovered in shellfish because of their high acute toxicity in mice upon intraperitoneal (*i.p.*) injections of lipophilic extracts. When they are present in shellfish at elevated levels, they rapidly kill mice, and they may interfere with the mouse bioassay (MBA) for okadaic acid (OA)-, brevetoxin (BTX)-, yessotoxin (YTX)- and azaspiracid (AZA)-group toxins (FAO/IOC/WHO, 2004).

In Europe SPXs have only recently been found in their producer dinoflagellate *A.ostenfeldii* in Scotland (John et al., 2003), Italy (Ciminiello et al., 2006), Denmark (MacKinnon et al., 2006) and in Ireland (Touzet et al., 2008). They have also been found in shellfish in Norway (Aasen et al., 2005), Spain (Villar González et al., 2006) and in Italy (Pigozzi et al., 2008). To date GYMs have not been reported in shellfish produced in Europe. They have only been detected in shellfish imported from Tunisia. Biré et al. (2002) and Marrouchi et al. (2009) reported GYM A and B in Tunisian clams. In Europe PnTXs have only recently been found in mussels in various parts of the Norwegian coast (Miles et al., 2010). Other European countries have not conducted surveys on PnTXs in shellfish to this end. PtTXs have not been reported in shellfish in Europe.

Although SPXs, GYMs, PnTXs and PtTXs are now known to occur in microalgae and/or shellfish in several parts of the world (Canada, Denmark, New Zealand, Norway, Scotland, Tunisia, USA and Japan), no information has been reported linking these toxin groups to poisoning events in humans (FAO/IOC/WHO, 2004; Cembella and Krock, 2008; Rhodes et al., 2010; Selwood et al., 2010).

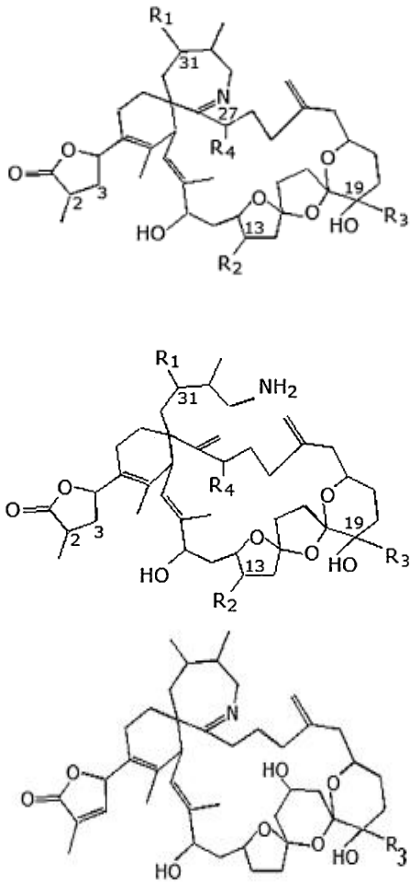
In addition to SPXs, GYMs, PnTXs and PtTXs, the CI group comprises prorocentrolides and spiro-prorocentrimines. Due to similarities in chemical structure and toxicity in mice these different groups of toxins have been grouped together. SPXs, GYMs and PnTXs are dealt with in this scientific opinion as they have been reported to occur in shellfish produced in Europe or imported into Europe (Cembella and Krock, 2008; Miles et al., 2010). Although PtTXs have not been reported in shellfish in Europe they are included in this opinion because recent findings suggest that they are biotransformation products of PnTXs in shellfish (Selwood et al., 2010). To date prorocentrolides and spiro-prorocentrimines have not been reported in shellfish in Europe.

2. Chemical characteristics

SPXs, GYMs, PnTXs and PtTXs, like other CIs, (prorocentrolides and spiro-prorocentrimines) are macrocyclic compounds with imine (carbon-nitrogen double bond) and spiro-linked ether moieties. Comparison of the chemical structures of the six CI toxin groups shows a high degree of structural similarity e.g. approximately 70 % homology between PnTXs and SPXs (Cembella and Krock, 2008). SPXs, PnTXs, GYMs and PtTXs are soluble in organic solvents such as methanol, acetone, chloroform and ethyl acetate.

SPXs are the largest group of the CIs and there are several studies on mass spectrometric (MS) and nuclear magnetic resonance (NMR) methods which have been used for structure elucidation of SPX analogues (Hu et al., 1995, 1996, 2001; Falk et al., 2001; Sleno et al., 2004a, b; Aasen et al., 2005; Sleno and Volmer, 2005; Ciminiello et al., 2006, 2007; MacKinnon et al., 2006; Krock and Cembella, 2007). Based on their chemical structure SPXs can be divided into three classes. The first, SPXs has a 6-5-5-polyether ring system in addition to the heptacyclic imine ring. This class includes SPXs A, B, C, D, 13-desmethyl SPX C (also known as SPX 1), 13,19-didesmethyl SPX C, 13-desmethyl SPX D and 27-hydroxy 13,19-didesmethyl SPX C (Figure 1). The second SPX class has the same 6-5-5-polyether ring system but the heptacyclic imine ring has opened to a keto amine (Figure 1). This class comprises SPX E and F which are shellfish metabolites of SPX A and SPX B, respectively. Because they have a hydrolysed cyclic imine group the SPX E and F are biologically inactive (Hu et al., 1996, 2001). The third class includes SPX G and its analogue 20-methyl SPX G (Figure 1). Both of them have a heptacyclic imine ring and an unusual 5-5-6-trispiroketal ring system which has not been found in other marine biotoxin groups (MacKinnon et al., 2006; Otero et al., 2010b). SPX C, SPX D, 13,19-didesmethyl SPX C, 13-desmethyl SPX C and SPX G, containing an additional methyl group on the imine ring when compared to SPX A and B, are suspected to be resistant to acidic and enzymatic hydrolysis in shellfish, and therefore they may be biologically active (Hu et al., 2001; Christian et al., 2008). It has also been reported that like other marine biotoxins e.g. okadaic acid (OA)-group toxins and pectenotoxin (PTX)-group toxins, SPXs can be metabolised in shellfish to fatty acid esters (Aasen et al., 2006; Doucet et al., 2007).

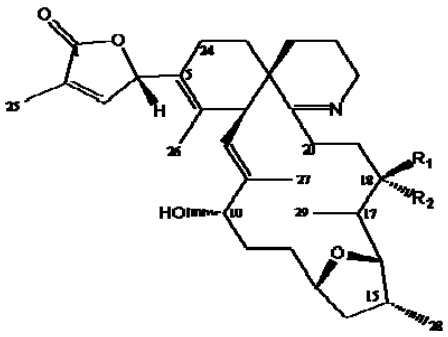
The structures of GYMs have also been characterised by applying NMR and MS techniques (Seki et al., 1995; Stewart et al., 1997; Miles et al., 2000; Miles et al., 2003). They are the smallest molecules of the group of CIs. The chemical structures of GYM A, GYM B and GYM C are known now (Figure 2). All GYM analogues have a six-membered imino ring. Their macrocycle contains 16 carbon units and one ether bridge. GYM-C is an oxidised analogue of GYM-A and is isomeric with GYM B at C-18. Possibly GYM C and GYM B originate from a precursor 18-deoxy GYM B, which is an analogue of GYM A formed through oxidation (Miles et al., 2000, 2003).



Spirolide (SPX)	R_1	R_2	R_3	R_4	$\Delta^{2,3}$	MW
SPX A	H	CH_3	CH_3	H	$\Delta^{2,3}$	691
SPX B	H	CH_3	CH_3	H		693
SPX C	CH_3	CH_3	CH_3	H	$\Delta^{2,3}$	705
SPX D	CH_3	CH_3	CH_3	H		707
13-desmethyl SPX C	CH_3	H	CH_3	H	$\Delta^{2,3}$	691
13,19-didesmethyl SPX C	CH_3	H	H	H	$\Delta^{2,3}$	677
13-desmethyl SPX D	CH_3	H	CH_3	H		693
27-hydroxy 13,19-didesmethyl SPX C	CH_3	H	H	CH_3	$\Delta^{2,3}$	691
SPX E	H	CH_3	CH_3	H	$\Delta^{2,3}$	709
SPX F	H	CH_3	CH_3	H		711
SPX G			H			691
20-methyl SPX G			CH_3			705

MW: molecular weight; $\Delta^{2,3}$: Double bond between C2-C3

Figure 1: Chemical structures of spirolides (SPXs) (modified from Otero et al., 2010b).



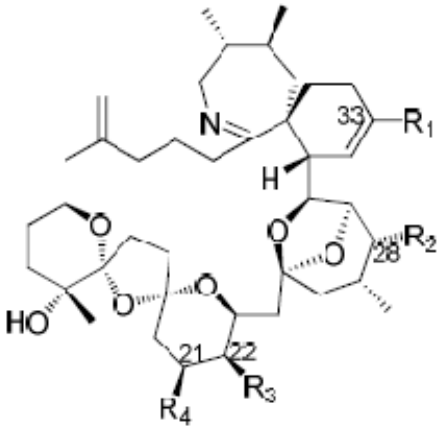
Gymnodimine (GYM)	R_1	R_2	MW
GYM A	H	- [(a)]	507
GYM B	H	OH	523
GYM C	OH	H	523

(a): Double bond between C17-C18; MW: molecular weight

Figure 2: Chemical structures of gymnodimines (GYMs) (modified from Cembella and Krock, 2008).

NMR and MS techniques have been applied to elucidate the chemical structures of PnTXs (Uemura et al., 1995; Chou et al., 1996a,b; Takada et al., 2001a; Selwood et al. 2010). Of the group of CIs the chemical structure of PnTXs is most closely related to that of SPXs. PnTXs have a 6-5-6 polyether ring system and they have an additional bicyclic ether moiety in the macrocycle (Figure 3). PnTX-analogues vary in the length of their cyclohexenyl side chains. While PnTX A has a carboxylic group at R1, PnTX B and PnTX C have a 2-amino acetic acid group at R1 and they are pairs of diastereoisomers at C-34. PnTX D has a C4 γ-ketobutyric acid moiety (Figure 3). For the novel PnTX-analogues E, F and G, Selwood et al., (2010) suggested that PnTX E has a carboxylic acid in the side chain and that it is the reduced analogue of PnTX D. The defined structure of PnTX E is as presented in Figure 3 (Selwood et al., 2010). The side chain of PnTX F was defined to have a lactone ring which readily hydrolyses to hydroxyl acid to form PnTX E. Selwood et al. (2010) suggested that the side chain of PnTX G was formed by an ethenyl group.

The latest results suggest that the novel PnTX F and G are progenitors of all the known PnTXs and PtTXs that are produced via metabolic transformation (e.g. hydrolysis) in shellfish (Selwood et al., 2010). It is probable that PnTX F is metabolised to PnTX D and E, while PnTX G is metabolised to PnTXs A-C and PtTXs A-C (Selwood et al., 2010).

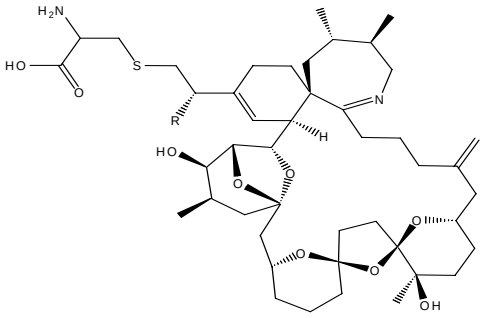


Pinnatoxin (PnTX)	R_1	R_2	R_3	R_4	MW
PnTX A	—CO_2H	OH	H	H	711
PnTX B, C	CO_2H, NH_2	OH	H	H	740
PnTX D	CO_2H, O	OH	H	H	781
PnTX E	CO_2H, OH	H	OH	CH_3	783
PnTX F	O, O	H	OH	CH_3	765
PnTX G		H	OH	CH_3	693

MW: molecular weight

Figure 3: Chemical structures of pinnatoxins (PnTXs) (modified from Miles et al. 2010; Selwood et al., 2010).

The chemical structures of the PtTX-analogues A, B and C were determined based on NMR and MS spectral analysis by Takada et al. (2001b) and Hao et al. (2006) (Figure 4). PtTXs and PnTXs are almost structurally identical with the difference that the cyclohexenyl side chain of PtTXs ends in a cysteine moiety. PtTXs have one acidic and two basic functional groups. PtTX B and C are epimers of each other at C-34. Just as PnTXs, PtTXs have a free carbocyclic group at the cyclohexenyl side chain while other CI compounds have lactones (Takada et al., 2001b; Cembella and Krock, 2008).



Pteriatoxin (PtTX)	R	MW
PtTX A	OH	830
PtTX B	CH_2-OH (R or S)	844
PtTX C	CH_2-OH (S or R)	844

MW: molecular weight; in brackets: R: rectus (clockwise) configuration; S: sinister (counter clockwise) configuration

Figure 4: Chemical structures of pteriatoxins (PtTXs) (modified from Cembella and Krock, 2008).

3. Regulatory status

Currently there are no regulations on CIs in shellfish in Europe or in other regions of the world. However, the toxicology working group of the EU Community Reference Laboratory for marine biotoxins (CRLMB) has proposed a guidance level of 400 µg sum of SPXs/kg shellfish meat (CRLMB, 2005; Pigozzi et al., 2008).

4. Methods of analysis

Several published methods exist for the determination of CIs. The mouse bioassay (MBA) is considered a simple way to detect CIs, but for ethical and scientific reasons there are growing concerns with respect to its use. Some functional assays and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have also been developed. None of the methods to determine SPXs, GYMs, PnTXs and PtTXs have been formally validated in interlaboratory validation studies.

4.1. Supply of appropriate reference material

Certified reference standards are available for 13-desmethyl SPX C and for GYM A (National Research Council Canada[13]), but there are no certified standards for other CIs. Certified reference materials are not available for shellfish containing SPXs, GYMs, PnTXs or other CIs.

4.2. Mouse bioassay

The MBA has traditionally been used to detect CIs in shellfish. SPXs were identified as a source of false positive results in the classical MBA developed by Yasumoto et al. (1978) for lipophilic toxins. Their signs of toxicity prompted the use of the term "fast acting" toxins (Cembella and Krock, 2008), to emphasise their action (neurological signs, convulsions, cramps) within a few minutes after the injection and death within 30 minutes. The reason for this response in the MBA is the high solubility of SPXs in polar organic solvents, such as methanol, aqueous ethanol or acetone, hence being readily incorporated into the same fraction as the other lipophilic toxins (Cembella and Krock, 2008; Otero et al., 2010 a,b). The classical MBA was also applied to identify GYM A for the first time in clams in Tunisia (Biré et al., 2002). The period of time when death occurred has not been reported for GYM A. Assuming that an extract from 25 g shellfish is injected into a 20 g mouse, the *i.p.* LD_{50} of 6.9 µg/kg b.w. for 13-desmethyl SPX C (Munday, unpublished studies; Munday, 2008) indicates a limit of

[13] http://www.nrc-cnrc.gc.ca/eng/index.html

detection (LOD) for this compound in the MBA of about 5.6 µg/kg shellfish. For SPX C, the *i.p.* LD_{50} of 8.0 µg/kg b.w. (Munday, unpublished studies; Munday, 2008) indicates an LOD in the MBA of about 6.4 µg/kg shellfish. For GYM A, the *i.p.* LD_{50} of 96 µg/kg b.w. (Munday et al., 2004), indicates an LOD of about 77 µg/kg shellfish in the MBA. For PnTx E, F and G, the *i.p.* LD_{50} values of about 50, 45 and 16 µg/kg b.w. (Selwood et al., 2010), indicate LODs in the MBA of 40, 36 and 13 µg/kg shellfish, respectively.

The main advantages of the MBA are:

- the provision of a measure of total toxicity based on the biological response of the animal to the toxin(s);
- it does not require complex or expensive analytical equipment.

The main disadvantages of the MBA are:

- it is not quantitative;
- it does not differentiate easily from high levels of other lipophilic marine biotoxins;
- no specific information is provided on individual toxins;
- it cannot be automated;
- it requires specialised animal facilities and expertise;
- the inherent variability in results between laboratories due to e.g. specific animal characteristics (strain, sex, age, weight, general state of health, diet, stress);
- it has not been validated;
- in many countries the use of the MBA is considered undesirable for ethical reasons.

4.3. Biochemical assays

The selective interaction of GYMs and SPXs with cholinergic receptors, where they act as blockers, has prompted the development of functional methods based on this interaction. Vilariño et al. (2009) developed an inhibition assay method based on the competition of GYM A and 13-desmethyl SPX C with fluorescent-labeled alpha-bungarotoxin for the nicotinic acetyl choline receptor (nAChR) of the electric organ of the electric ray *Torpedo marmorata*, with quantification using fluorescence polarisation. Whereas the method was developed using GYM A and 13-desmethyl SPX C, it is selective for any analogue of CIs that may interact with nAChR. The method has been shown to be adequate for the analysis of GYM A and 13-desmethyl SPX C in various shellfish species such as mussels, clams, cockles and scallops as reported by Fonfría et al. (2010). The limit of quantification (LOQ) of the method is 80 µg/kg for GYM A and 85 µg/kg for 13-desmethyl SPX C in shellfish. The applicability of the receptor-based fluorescence polarisation method for the other CIs than GYM A and 13-desmethyl SPX C has not been reported. The binding of GYM A and 13-desmethyl SPX C to acetylcholine-binding proteins (AchBPs) in two snail species: *Aplysia californica* and *Lymnaea stagnalis* has also been shown recently using stopped-flow instrumentation and conventional binding competition (Bourne et al., 2010). This AChBP-based binding assay could also be used to quantitatively detect CIs in shellfish but more research is needed.

The main advantages of the receptor-based fluorescence polarisation method are:

- it is technically easy to perform;
- the necessary equipment is inexpensive;
- the method provides fast results;
- it detects any analogue of the CIs that may interact with nAChR.

The main disadvantages of the receptor-based fluorescence polarisation method are:

- it is dependent on availability of receptors from *Torpedo marmorata*;
- the method has not been validated.

4.4. Chemical methods

The imino moiety of CI-molecules is a very good proton acceptor and therefore LC-MS/MS methods can detect CIs at low levels and have high selectivity. LC-MS/MS methods are currently the methods of choice for analysis of SPXs, GYMs, PnTXs and PtTXs in shellfish. CIs lack chromophores and therefore the optical detection methods such as ultraviolet (UV) do not provide sufficient selectivity. Recently multitoxin LC-MS/MS methods have been developed for comprehensive phycotoxin monitoring of lipophilic marine biotoxins (Fux et al, 2007; Gerssen et al., 2009a,b).

4.4.1. LC-MS/MS methods

Several publications have reported LC-MS/MS methods for SPXs, GYMs, PnTXs and PtTXs (Cembella et al., 1999; Quilliam et al., 2001; Stirling, 2001; Takada et al., 2001a, b; Biré et al., 2002; MacKenzie et al., 2002; Aasen et al., 2005; Ciminiello et al., 2006; Villar González et al., 2006; Fux et al., 2007; Gerssen et al., 2009a, b; Álvarez et al., 2010, Miles et al., 2010; Selwood et al., 2010). These methods are based on reversed-phase LC coupled with electrospray ionization MS used in either the selected ion monitoring (SIM) or selected reaction monitoring (SRM) modes.

Shellfish tissues are usually extracted with (aqueous) methanol. If necessary, the crude extract may be further partitioned with chloroform. Alternatively, a solid phase extraction (SPE) procedure can be used for purification. In order to reduce the matrix effect from shellfish in the LC-MS/MS analysis, SPE purification prior to acidic and basic HPLC separation conditions were studied for several lipophilic marine biotoxins including 13-desmethyl SPX C and GYM A by Gerssen et al. (2009a, b). The reported recoveries of the SPE purification for 13-desmethyl SPX C and GYM A in mussel extract were ≥90 % (Gerssen et al., 2009a) and LODs were reported to be 0.8 µg/kg shellfish meat for 13-desmethyl SPX C and 3.7 µg/kg shellfish meat for GYM A (Gerssen et al., 2009b). LODs vary with procedures and LC-MS/MS instruments used. Higher LODs for multiresidue methods were reported for GYM A (at levels 10-20 µg/kg shellfish) (MacKenzie et al. (2002) and for 13-desmethyl SPX C (approximately 7 µg/kg shellfish) (Álvarez et al., 2010). LODs for the LC-MS/MS methods used for analysis of PnTXs in shellfish were not reported, but the lowest concentration measured for PnTX G was reported to be 5 µg/kg shellfish (Miles et al., 2010). For the LC-MS/MS method used for structural characterisation of PtTXs isolated from shellfish, the LODs were not determined (Takada et al., 2001b).

An inter-laboratory study of an LC-MS/MS method for determination of various lipophilic toxins in shellfish extracts was carried out by McNabb et al. (2005). The method was first subjected to a full

single-laboratory validation and then to a limited interlaboratory study between 8 laboratories in 7 countries. GYM A, SPX A, SPX D and 13-desmethyl SPX C were measured in the study but the method was quantitative only for GYM A. For SPXs the relative response factors were used to estimate their concentrations. The LOD for GYM A was 1 µg/kg shellfish meat. The between-laboratories reproducibility values for lipophilic toxins including GYM A were compared with the Horwitz criterion and ranged between $HORRAT_R$ values of 0.8 to 2.0[14] (McNabb et al., 2005).

The main advantages of the LC-MS/MS methods are:

- they are very specific and thus superior for use as confirmatory methods;
- their LODs for SPXs, GYMs and PnTXs are lower than with the other methods.

The main disadvantages of LC-MS/MS methods are:

- they require costly equipment and highly trained personnel;
- there is a lack of reference materials of the various analogues of SPXs, GYMs, PnTXs and PtTXs;
- no formal interlaboratory validation studies have been published for GYMs, SPXs, PnTXs or PtTXs and detailed performance characteristics have not been reported.

4.4.2. HPLC-UV method

Recently a reversed-phase HPLC-UV method was developed for routine analysis of GYMs in shellfish (Marrouchi et al., 2009). UV detection at wavelength of 210 nm was used to detect GYM A and GYM B in clam samples. The LOD was reported to be 2.4 µg GYM A/kg digestive gland of clams and the results were confirmed by LC-MS/MS. No other HPLC methods applying optical detection techniques either for GYMs, SPXs, PnTXs or PtTXs were identified in the literature.

The main disadvantages of the HPLC-UV method are:

- due to low UV absorption of CIs the method is not specific and confirmation by LC-MS/MS is needed;
- there is a lack of reference materials of the various analogues of SPXs, GYMs, PnTXs and PtTXs;
- no formal interlaboratory validation studies have been carried out for SPXs, GYMs, PnTXs or PtTXs, and detailed performance characteristics have not been reported.

[14] $HORRAT_R$: The observed relative standard deviation calculated from results generated under reproducibility conditions (RSD_R) divided by the RSD_R value calculated from the Horwitz equation. The Horwitz equation is a generalised precision equation which has been found to be independent of analyte and matrix but solely dependent on concentration for most routine methods of analysis.

4.5. Proficiency tests

For CIs there are no ongoing proficiency tests.

4.6. Summary of methods

The MBA has traditionally been used to detect CIs in shellfish but for reasons of animal welfare there is growing concern with respect to its use and it has shown poor specificity as other lipophilic toxins interfere in the analysis. The receptor-based fluorescence polarisation method has been shown to be able to detect GYM A and 13-desmethyl SPX C at relevant levels in shellfish. CIs lack chromophores and therefore the optical detection methods such as ultraviolet HPLC-UV methods do not provide sufficient selectivity. Therefore recent studies have focussed primarily on the development of multiresidue LC-MS/MS methods for determination of SPXs, GYMs and PnTXs in shellfish. Some of these studies strongly suggest that the LC-MS/MS methods would be of value for the quantification of SPXs, GYMs, PnTXs and PtTXs. However, the optimisation of these methods, their (inter-laboratory) validation and the development of standards and reference materials are necessary.

5. Occurrence of CIs

5.1. Data collection

Following a request by the European Food Safety Authority (EFSA), France, Italy, the Netherlands and Spain provided data on the occurrence of CIs in shellfish. The results for a total of 1821 samples analysed by LC-MS/MS were submitted. The number of samples differs considerably between the reporting countries, ranging between 20 samples from Spain and 852 samples from France. The reported results are over the years 2002-2008. Although some small surveillance studies have been conducted in the four countries, GYMs have not been detected. Hence they were not included in the data analysis and statistics were calculated only for SPXs. Very recently PnTX G and A have been found in blue mussels in Norway (Miles et al., 2010). In addition Miles et al., (2010) reported occurrence results for SPXs in Norway. These data were, however, not available for inclusion in the set of occurrence data submitted to EFSA. Table 2 shows a summary of the number of data submitted by four European countries including the reason for testing, analytical method applied, LOD and LOQ of the methods.

Table 2: Data submissions from European countries for SPXs in the period from 2002 to 2008.

Country	Year(s) of harvesting	Number of samples	Purpose of testing[(a)]	Method of testing	LOD (µg/kg)	LOQ (µg/kg)
France	2005-2008	852	PreMC	LC-MS/MS	1	1.6
Italy	2002-2008	635	PreMC	LC-MS/MS	2	5
The Netherlands	2007	314	PreMC	LC-MS/MS	0.8-1.1	2.4-3.4
Spain	2007-2008	20	PreMC	LC-MS/MS	20	70
Total	**2002-2008**	**1821**			**0.8-20**	**1.6-70**

Pre-MC: pre-market control; LOD: limit of detection; LOQ: limit of quantification
(a): PreMC are samples collected at the place of origin, before or during harvesting.

The LOD and LOQ values for the LC-MS/MS datasets refer to 13-desmethyl-SPX C, the only SPX for which a certified calibrant solution is available. France, Italy and the Netherlands reported similar LOD and LOQ values, whereas Spain reported LOD and LOQ more then ten fold higher than the other countries, probably due to different method and different equipment. Because of the high LOD

and LOQ values and absence of any quantified values in the data from Spain, they were excluded from the further calculations.

5.2. CI concentration in shellfish

5.2.1. SPXs

Normally the whole shellfish is consumed and therefore the occurrence data for SPXs need to be expressed as whole shellfish meat. Most of the analyses were performed on whole shellfish meat. In a few samples only hepatopancreas was analysed, in which case a factor of 5 was used to convert the value to whole shellfish meat. This factor, though not representing exactly all individual shellfish species, is considered to represent a good approximation.

A total of 1801 samples from three countries were considered for the initial descriptive statistical calculations by country. The "bounding" approach was applied for values reported below LOD or LOQ in order to identify the possible range of the data. The lower bound (LB) is obtained by assigning a value of zero to all the samples reported as <LOD or <LOQ. The upper bound (UB) is obtained by assigning the value of the LOD to values reported as <LOD and the value of the LOQ to values reported as <LOQ. The comparison between UB and LB values shows the impact of the number of non-detected samples on the concentration estimate.

13-Desmethyl-SPX C was the SPX-analogue most often reported by the three submitting countries. Of the other SPX-analogues, only SPX A, SPX B and 13-desmethyl SPX D were occasionally reported. The data are expressed as sum of SPXs. Based on the reported data 13-desmethyl-SPX C appears to be the main contributor to this sum. Where other SPXs were reported they were quantified based on the 13-desmethyl-SPX C calibrant (assuming equal responses). An overview of the basic statistics for the occurrence of SPXs in France, Italy and the Netherlands in the years 2002-2008 is provided in Table 3.

Table 3: Statistics of the occurrence data of SPXs in shellfish analysed by LC-MS/MS over the period from 2002 to 2008 by member states.

Country	N	Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum	% of samples not quantified[(a)]
		µg sum of SPXs/kg shellfish meat				
France	852	0/1.6	3.2/3.9	14.5	90	61 %
Italy	635	0/5	1.2/6	0/5	105	97 %
The Netherlands	314	0/0.8	0.2/1.1	0/1.1	9.6	96 %
Total	**1801**	**0/3**	**3/4.1**	**8.9**	**105**	**80 %**

N: number of samples; P95: 95th percentile; UB: upper bound; LB: lower bound
No information was available on measurement uncertainty. When two values are given it indicates the respective LB or UB values for samples below the LOD or LOQ. The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting "<LOD" with LOD value and "<LOQ" with LOQ value. LOD and LOQ are those defined for the specific single analysis.
In the presence of a low level of contamination and a very high percentage of non detected results, median, mean, and in some cases even the 95th percentile are influenced by the choice of UB or LB approach.
(a): "Not quantified" means no numerical value reported.

The basic statistics indicate an amount of SPXs in the dataset provided by three European countries ranging from "not detected" to 105 µg sum of SPXs/kg. The percentage of analytical results <LOD or <LOQ and therefore without a quantified value varies between countries, ranging from 61 % in

France (years 2005-2008) to 97 % in Italy (years 2002-2008). The percentage of not quantified results in all 1801 samples is 80 %.

Occurrence data on other SPX analogues in shellfish than those included in the data submission to EFSA have been reported for SPX C and 20-methyl SPX G in Norway (Aasen et al., 2006; Miles et al., 2010). For SPX C 56 % of the 166 mussel samples analysed, gave positive results with the typical concentration range of 4-25 µg/kg shellfish. Of the same samples, 78 % were positive for 20-methyl SPX G and the typical concentrations varied between 4 and 20 µg/kg shellfish. About 20 % of the samples contained 13-desmethyl SPX C at the typical concentrations of 20-50 µg/kg shellfish. Maximum concentrations 49, 38 and 226 µg/kg shellfish were reported for SPX C, 20-methyl SPX G and 13-desmethyl SPX C, respectively (Miles et al., 2010).

5.2.2. PnTXs

Recent preliminary results from a range of shellfish samples from the monitoring programme of the Norwegian Food Safety Authority showed that whilst only low levels of PnTX A (level not stated) were detected, PnTX G was detected in mussel samples at typical concentrations in the range of 5 to 30 µg/kg, but also higher levels up to 115 µg/kg were found. There was no sign of fatty acid esters of PnTXs in Norwegian mussels (Miles et al., 2010). PtTXs have not been detected in shellfish in Europe.

5.3. Difference between shellfish species

The distribution of SPXs in different shellfish species was evaluated based on results of 1801 samples. The resulting statistical descriptors are shown in Table 4.

Table 4: Statistical descriptors for occurrence of SPXs in different shellfish species reported by France, Italy and the Netherlands.

Species	N	Total concentration of SPXs µg sum of SPXs/kg shellfish meat				% of samples not quantified[(a)]
		Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum	
clams	146	0/1.6	1.7/3.1	7.1	28	68 %
cockles	34	0/1.1	0.5/1.5	-	13	91 %
gastropods	2	4.5/7	5/7	-	9	50 %
mussels	1305	0/5	2.0/5	8.6	105	82 %
oysters	272	0/1.1	2.3/3.2	15	47	72 %
scallops	42	-	-	-	-	100 %
All Species	**1801**	**0/3**	**2/4.1**	**8.9**	**105**	**80 %**

N: number of samples; P95: 95th percentile; UB: upper bound; LB: lower bound
For most of the data no information was available on measurement uncertainty. When two values are given it indicates the respective LB or UB values for samples below the LOD or LOQ. The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting "LOD" with LOD value and "LOQ" with LOQ value. LOD and LOQ are those defined for the specific single analysis.
In the presence of a low level of contamination and a very high percentage of non detected results, median and mean are influenced by the choice of UB or LB approach.
(a): "Not quantified" means no numerical value reported.

In mussels, oysters and clams, for which a sufficient number of samples is available, the 95th percentile of the concentration of SPXs is about 9, 15 and 7 µg sum of SPXs/kg shellfish meat, respectively. In scallops SPXs were not detected.

5.4. Influence of processing

There are no data on the influence of processing on the levels of CIs in shellfish.

6. Human consumption of shellfish

As in the previous scientific opinions on marine biotoxins, the Panel on Contaminants in the Food Chain (CONTAM Panel) used a consumption of 400 g of shellfish meat in one meal to represent a large portion size.[15]

7. Exposure assessment

7.1. Deterministic estimate of dietary exposure to SPXs

CIs are not regulated at the moment therefore potentially all contaminated product could reach the market although the control for the regulated marine biotoxins using the MBA might detect samples containing high levels of CIs. Using the occurrence data officially submitted by France, Italy and the Netherlands (2002-2008) and assuming a scenario where all shellfish for which analyses were reported reach the market, the 95th percentile of 1801 samples is 8.9 µg sum of SPXs/kg shellfish meat (see Table 3). Consumption of a 400 g portion of shellfish meat containing SPXs at the 95th percentile would result in a single dietary exposure of 0.06 µg sum of SPXs/kg b.w. for 60 kg person (Table 5).

Table 5: Deterministic dietary exposure estimate of SPXs expressed as sum of SPXs based on available results.

P95 of occurrence	8.9 µg sum of SPXs/kg shellfish meat
Exposure by eating a 400 g portion	3.6 µg sum of SPXs/person (0.06 µg sum of SPXs/kg b.w.)

P95: 95th percentile; b.w.: bodyweight

7.2. Probabilistic estimate of dietary exposure to SPXs

A probabilistic estimate of dietary exposure to SPXs has been performed by a Monte Carlo simulation using the distributions of both the occurrence data and the data on the consumption of shellfish.

[15] The EFSA Journal (2008), 589, 1-62. Available from http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_589_okadaic_acid_en.pdf?ssbinary=true
The EFSA Journal (2008), 723, 1-52. Available from <http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_723_AZA_en,0.pdf?ssbinary=true>
The EFSA Journal (2008), 907, 1-62. Available from http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej_907_yessotoxin_en.pdf?ssbinary=true
The EFSA Journal (2009), 1019, 1-76. Available from <http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1019_saxitoxin_marine_biotoxins.pdf?ssbinary=true>
The EFSA Journal (2009), 1109, 1-47. Available from http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1109_pectenotoxins_en.pdf?ssbinary=true
The EFSA Journal (2009), 1181, 1-61. Available from http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1181_domoic%20acid_marine%20biotoxins_en.pdf?ssbinary=true
The EFSA Journal (2009), 1393, 1-38. Available from <http://www.efsa.europa.eu/en/scdocs/doc/1393.pdf>

Compared to the deterministic estimate the probabilistic exposure estimate provides information on the probability to exceed a specific exposure level.[16]

Because a person eating shellfish will not eat the same portion size containing the same level of toxins each time, the probabilistic calculation includes all the combinations of all different occurrence and consumption data. For the probabilistic estimate the same occurrence data obtained by the LC-MS/MS measurements of the samples as used in the deterministic estimate (Table 3) were used. Because insufficient information is available on the distribution of portion sizes, the CONTAM Panel decided to use a triangular distribution as a simple and pragmatic approach. A triangular distribution is characterised by three values, the minimum, the most probable (modal value) and the maximum. In the case of shellfish consumption a value of 0 g was used as a minimum. As in the previous scientific opinions, from the range of 10 g to 136 g reported as mean consumption figures, the CONTAM Panel chose a value of 100 g to be used as "most probable" value, although there is no evidence that it is the most frequently consumed portion. As in the previous opinions, a portion size of 400 g of shellfish meat was used to represent the maximum[15].

The probabilistic dietary exposure estimate is presented in Figure 5 illustrating the chance of a specific level of exposure to sum of SPXs when consuming a single portion of shellfish. The exposure distribution has a median value of 0.44 µg of sum of SPXs per portion, a mean of 0.92 µg of sum of SPXs per portion and a 95th percentile of approximately 3.4 µg of sum of SPXs per portion, equal to 0.007, 0.015 and 0.06 µg sum of SPXs/kg b.w. for a 60 kg person, respectively. The chance to exceed the deterministic dietary exposure estimate of 3.6 µg sum of SPXs per person corresponding to a consumption of a portion of 400 g of shellfish containing the 95th percentile SPX concentration, is about 4 %.

[16] All samples with quantified levels (>LOQ) of SPXs were submitted to the best fit approach of the @RISK tool, to obtain an optimal adaptation of the distribution function, as described in the following: RiskLognorm(7,9566;15,668;RiskShift(1,3452);RiskTruncate(5;)) The distribution function was truncated at the highest LOQ of 5 µg/kg as shown in the formula. The values below LOQ were characterised as follows: a random assignment of the values was performed using a discrete distribution [RiskDiscrete ({0;1};% <LOQ; % >LOD) to reflect the number of samples at or below the LOQ and the number of samples with quantified toxin concentrations. This means that the ratio of non-quantified/quantified samples was adjusted to the different data. These latter data were simulated using a uniform distribution function [*RiskUniform (0;LOQ)*].

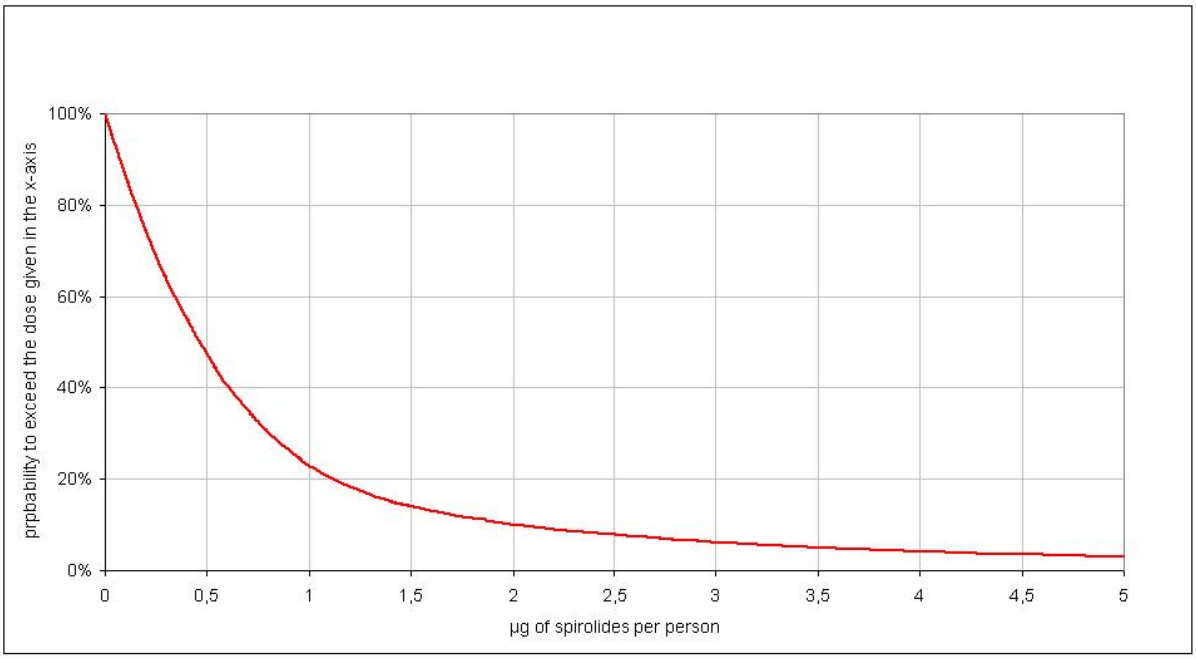


Figure 5: Probability of dietary exposure to spirolides (SPXs) expressed as sum of SPXs resulting from consumption of a single portion of shellfish.

8. Toxicokinetics

Specific information on the absorption, distribution, metabolism and excretion of SPXs, GYMs or PnTXs in laboratory animals or humans is not available from the literature. However, systemic toxicity observed following oral administration (gavage) for several CIs clearly indicate that these compounds are absorbed from the intestinal tract (Richard et al., 2001; Munday et al., 2004; Munday, 2008). Recent results from an on-going EU research project (Conffidence report, 2010) show that 13-desmethyl SPX C and 13,19-didesmethyl SPX C can be detected in blood, urine and faeces of mice following oral administration. Fifteen minutes after a single oral dose of 27.9 µg/kg b.w. of 13-desmethyl SPX C and 32.2 µg/kg b.w. of 13,19-didesmethyl SPX C both compounds were detected in blood at concentrations of 0.28 and 0.20 ng/mL, respectively. No quantifiable levels were found in blood after 1 hour and 24 hours (LOQ not stated). After 1 hour urinary concentrations for 13-desmethyl SPX C and 13,19-didesmethyl SPX C were 1.4 and 1.0 ng/mL, respectively, but were below the LOQ after 24 hours (Conffidence report, 2010). The rapid recovery seen in animals following a sub-lethal dose of GYM A (Munday et al., 2004) or 13-desmethyl SPX C (Richard et al., 2001; Munday et al., 2004) suggests a rapid detoxification or excretion of the toxins in animal species (FAO/IOC/WHO, 2004).

No information on the biotransformation of CIs in mammals have been identified, however, it is known that these compounds can be bio-transformed, including detoxification by reduction (GYM) or by ring opening (SPX A and B) in molluscs (Hu et al., 1996; Stewart et al., 1997; Munday, 2008). Whether such pathways operate in mammals is not known.

9. Toxicity data

9.1. Mechanistic considerations

SPXs and GYMs are neurotoxic and have a similar mechanism of toxicity as indicated by the similarity of the signs of intoxication after *i.p.* injection to mice. The intact cyclic imine-group is assumed to be responsible for the neurotoxicity of SPXs and GYMs (Munday, 2008; Bourne et al., 2010). There is evidence that the neurotoxic action of SPXs and GYMs is based on their inhibition of both the muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR) in the central- and peripheral nervous system (Munday, 2008). Because PnTXs cause respiratory paralysis in mice, it is likely that they also target the nAChR at the neuromuscular junction (Selwood et al., 2010), and recently it has been confirmed that they bind to nAChR (Miles et al., 2010). Mechanistic data for the PtTXs are currently not available.

For GYM A, paralysis of the hind legs and severe dyspnoea observed in animals, are likely to occur via blockade of the endplate nAChR at the neuromuscular junction of skeletal muscles. It has been found that signs induced by *i.p.* injection of GYM resemble those induced by the nAChR antagonist tubocurarine. Moreover, the acetylcholinesterase inhibitor neostigmine inhibits GYM-induced toxicity (Biré et al., 2002; Munday et al., 2004; Kharrat et al., 2008). At subnanomolar concentrations GYM A inhibits twitch responses in isolated mouse phrenic hemidiaphragm and blocks both neuronal and muscular nAChRs in electrophysiological assays in a concentration- and time-dependent manner (Kharrat et al., 2008). GYM-A inhibited the specific binding of alpha-bungarotoxin to nAChRs confirming that both groups of CIs target muscular and neuronal nAChR subtypes with high affinity (Kharrat et al., 2008). Binding of 13-desmethyl SPX C and GYM A to nAChRs has also been confirmed in binding assays and voltage-clamp recordings on muscle and neuronal nAChR revealing potent antagonism for both types of receptors (Bourne et al., 2010). The main difference between GYMs and SPXs is that GYMs show a reversible effect, whereas binding of SPXs seem to be irreversible (Molgó et al., 2007, 2008; Vilariño et al., 2009; Fonfría et al., 2010). 13-Desmethyl SPX C inhibited mAChRs in human neuroblastoma cells through the blockage of ACh-elicited calcium signals and the long-term reduction of membrane-associated mAChRs (Conffidence report, 2010).

9.2. Effects in laboratory animals

9.2.1. Acute toxicity

The acute toxicity of CIs is characterised by the rapid onset of systemic neurotoxicity following *i.p.* injection in mice and death within minutes. CIs are therefore often denoted “fast acting toxins”.

9.2.1.1. Toxicity following intraperitoneal (*i.p.*) administration

SPXs

Data on the acute *i.p.* toxicity of SPXs are summarised in Table 6. The most toxic SPXs are 13-desmethyl SPX C, SPX C, and 20-methyl SPX G with LD_{50} values between 6.9 and 8.0 µg/kg b.w. (Munday, 2008). The toxicity of a mixture of SPXs, containing predominantly 13-desmethyl SPX C, was reported to be lower with a LD_{50} of 40 µg/kg b.w. (Richard et al., 2001; Munday, 2008). SPXs E and F, in which the cyclic imine moiety is opened, are much less toxic with no effects observed up to a dose of 1000 µg/kg b.w. Mice receiving lethal doses of SPXs died between 3 and 20 minutes after dosing. If the animals survived for more than 20 minutes, they recovered fully, and their subsequent appearance and behaviour were normal (Munday, 2008).

Purified 13-desmethyl SPX C was administered at 75, 260 and 2000 µg/kg b.w. to female CD-1 mice and 2000 µg/kg b.w. to Sprague Dawley rats. All mice died within 8, 4 and 2 minutes, respectively, and the rats within 2 minutes. After *i.p.* administration of 13-desmethyl SPX C to adult rats and mice, a complex cascade of clinical signs was observed, which were less severe in rats than in mice at the equivalent dose of the 13-desmethyl SPX C. The severe neurotoxic symptoms included moderate hunched up appearance, decreased movements and exploratory behaviour, abdominal breathing, respiratory distress and -arrest, contractions in front legs, tremors involving the entire body resembling a seizure, exophthalmia with increased lacrimation and urination immediately before death (Gill et al., 2003).

Table 6: Acute toxicity of SPXs after intraperitoneal (*i.p.*) injection in mice (modified and updated from Munday, 2008).

Compound	Dose (µg/kg b.w.)	Parameter	Reference
SPX mixture (crude extract)	40	LD_{50}	Richard et al. (2001)
SPX B	250	LD_{100}[(a)]	Hu et al. (1995)
SPX D	250	LD_{100}[(a)]	Hu et al. (1995)
SPX C	8.0 (CI 4.6-16.2)	LD_{50} (fed mice)	Munday (2008)
SPX E	1000	no effect observed	Hu et al. (1996)
SPX F	1000	no effect observed	Hu et al. (1996)
13-desmethyl SPX C	6.9 (CI 5-8)	LD_{50} (fed and fasted mice)	Munday (2008)
13-desmethyl SPX C	27.9	LD_{50}	Conffidence report (2010)
20-methyl SPX G	8.0 (CI 3.9-14.1)	LD_{50} fed mice	Munday (2008)
13,19-didesmethyl SPX C	32.2	LD_{50}	Conffidence report (2010)

CI: 95 % confidence interval; (a): No information is provided on how the LD_{100} was derived or if other doses were tested.

GYMs

Studies of the acute *i.p.* toxicity of GYMs are summarised in Table 7. GYM A is highly acutely toxic to female Swiss mice by *i.p.* injection with an LD_{50} of 80-96 µg/kg b.w. Signs of acute toxicity of GYM A and GYM B included hyperactivity, jumping, followed by slower movements, paralysis and extension of the hind legs. The mice subsequently became completely immobile and unresponsive to stimuli. Respiratory distress was apparent, with marked abdominal breathing. The respiratory rate progressively decreased, until respiration ceased altogether. Pronounced exophthalmia was observed shortly before death, which invariably occurred within 15 minutes of injection. No macroscopic abnormalities were recorded at necropsy. At toxic, but sub-lethal dose levels, prostration and respiratory distress were recorded, but the mice recovered within 30 minutes to an apparently normal state, and no adverse effects were observed during a subsequent 21-day observation period (Munday et al., 2004). GYM A was 10-fold more toxic than GYM B with mean LD_{50} values after *i.p.* injection of 80 and 800 µg/kg b.w., respectively, reported by Kharrat et al. (2008). The LD_{50} for GYM A was remarkably low after intra-cerebroventricular (*i.c.*) injection (3 µg/kg b.w.). This observation that GYM A was about 30 times more toxic when administered *i.c.* than by *i.p.* injection, indicates that it acts on both the central and peripheral nervous system (Kharrat et al., 2008).

PnTXs

Acute toxicity studies for PnTXs with *i.p.* injection in mice are summarised in Table 7. LD_{50} values ranged between 16 and 50 µg/kg b.w. with PnTX E and F being the most toxic analogues (Rhodes et al., 2010; Selwood et al., 2010). Takada et al. (2001a) suggested that the toxicity is dependent on the stereochemistry of the analogues.

Table 7: Acute toxicity of GYMs, PnTXs and PtTXs after intraperitoneal (*i.p.*) injection in mice (modified and updated from Munday, 2008).

Compound	Dose (µg/kg b.w.)	Parameter	Reference
GYM A (crude extract)	450	LD_{50}	Seki et al. (1995)
GYM A (crude extract)	700	MLD	Stewart et al. (1997)
GYM A (> 95 % pure)	96 (CI 79-118)	LD_{50}	Munday et al. (2004)
GYM A	80	LD_{50}	Kharrat et al. (2008)
GYM B	800	LD_{50}	Kharrat et al. (2008)
(+)-PnTX A	135	LD_{99}	Uemura et al. (1995)
(+)-PnTX A	180	LD_{99}	McCauley et al. (1998)
(-)-PnTX A (synthetic)	5000	no effect observed	McCauley et al. (1998)
PnTXs B and C[(a)]	22	LD_{99}	Takada et al. (2001a)
PnTX D	400	LD_{99}	Chou et al. (1996a)
PnTX E	45 (CI 32-58)	LD_{50}	Selwood et al. (2010)
PnTX F	16	LD_{50}	Selwood et al. (2010)
PnTXs E and F[(b)]	13 (CI 12.5-15.8)	LD_{50}	Rhodes et al. (2010)
PnTX G	50 (CI 35-66)	LD_{50}	Selwood et al. (2010)
PtTX A	100	LD_{99}	Takada et al. (2001b)
PtTX B and C[(a)]	8	LD_{99}	Takada et al. (2001b)

CI: 95 % confidence interval; MLD: minimum lethal dose; (+) and (–) refer to stereoisomers of PnTX A molecule; (a): 1:1 mixture of B and C; (b): Estimated from the LD_{50} of algal extract containing approximately 10 µg PnTX/mg (Rhodes et al., 2010).

At lethal doses of PnTXs, mice were hyperactive for up to 10 minutes followed by an abrupt decrease in activity, abdominal breathing, and extension of the hind legs and, in some cases, slight exophthalmia although respiration rate was still normal. In the next 2-3 minutes, respiration rate precipitately declined, and between 22 and 26 minutes after dosing, mice died with a brief period of running movements just before death associated with severe exophthalmia. At death, the hind legs were fully extended. At sub-lethal doses, mice showed abdominal breathing and became very lethargic 9-13 minutes after dosing; respiration rate remained normal, however, and full recovery was achieved within 2 hours. Behaviour and appearance remained normal throughout a subsequent 14-day observation period and no abnormalities were noted at necropsy, and organ weights were within the normal range (Selwood et al., 2010).

There is evidence that PnTX G transforms to PtTX A and B/C (see Chemical characteristics). Limited data using the MBA revealed that a mixture of PtTX B/C was 12-fold more toxic than PtTX A with respective LD_{99} values of 8 versus 100 µg/kg b.w. (Takada et al., 2001b).

9.2.1.2. Toxicity following oral administration

SPXs

Oral toxicity studies of the SPX analogues administered by gavage and in food are presented in Table 8. SPXs showed higher toxicity in studies by the gavage route compared to administration on food. By gavage, the LD_{50} of the most toxic forms: SPX C, 13-desmethyl SPX C and 20-methyl SPX G ranged between 53 and 176 µg/kg b.w. Overall, in feeding experiments LD_{50} values ranged from 500 to 1005 µg/kg b.w. with values of 500-780 µg/kg b.w. for SPX C, 13-desmethyl SPX C and 500-625 µg/kg for 20-methyl SPX G. Signs of toxicity were similar to those observed after *i.p.*

injection (Munday, unpublished studies) (Munday, 2008). In general the LD_0 values (i.e. a dose resulting in no deaths) are close to the LD_{50} values, indicating a steep dose response relationship.

Table 8: Acute toxicity of SPXs, GYMs and PnTXs after oral administration in mice.

Compound	Route	Dose (µg/kg b.w.)	Parameter	Reference
SPX (crude extract)	gavage	1000	LD_{50}	Richard et al. (2001)
SPX C	gavage	176	LD_{50} fed	Munday (2008)
	gavage	53 (CI 50-63)	LD_{50} fasted	
	fed on cream cheese	625 780	LD_0 fed LD_{50} fed[(c)]	
	fed on cream cheese	400 500 (CI 353-657)	LD_0 fasted LD_{50} fasted[(c)]	
13-desmethyl SPX C	gavage	157 (CI 123-198)	LD_{50} fed	Munday (2008)
		125 (CI 87-166)	LD_{50} fasted	
13-desmethyl SPX C	fed on pellet of mouse food	625 (CI 547-829)	LD_{50} fasted[(a)]	Munday (2008)
		591 (CI 500-625)	LD_{50} fasted[(b)]	
	fed on cream cheese	780 1005 (CI 861-1290)	LD_0 fed LD_{50} fed[(c)]	
		400 500 (CI 381-707)	LD_0 fasted LD_{50} fasted[(c)]	
20-methyl SPX G	gavage	157	LD_{50} fed	Munday (2008)
		88 (CI 27-120)	LD_{50} fasted	
20-methyl SPX G	fed on cream cheese	500 625 (CI 476-882)	LD_0 fed LD_{50} fed[(c)]	Munday (2008)
		400 500 (CI 381-707)	LD_0 fasted LD_{50} fasted[(c)]	
GYM A (> 95 % pure)	gavage	755 (CI 600-945)	LD_{50}	Munday et al. (2004)
	pipetted into the mouth	4057 (CI 3750-4390)	LD_{50}	
	voluntary feeding on mouse food	7500	no effect observed	
PnTXs E and F	gavage	23 (CI 20-25)	LD_{50}[(d)]	Rhodes et al. (2010)
PnTXs E and F	voluntary feeding on cream cheese	60 (CI 45-85)	LD_{50} fasted[(d)]	Rhodes et al. (2010)

CI: 95% confidence interval; ND; not determined; (a): Solution of pure toxin administered with dry mouse food to mice after overnight fast; (b): Solution of pure toxin administered with mixed pellet of moist mouse food; (c): Solution of pure toxin administered with cream cheese; (d): Estimated from the LD_{50} of algal extract containing approximately 10 µg PnTX/mg (Rhodes et al., 2010).

GYMs

The oral LD_{50} value for GYM A obtained by gavage was 8-fold higher than that with *i.p.* injection in mice (755 µg/kg b.w.). No signs of toxicity were observed in mice ingesting GYM A in food at a dose about 7500 µg/kg (Munday et al., 2004) (Table 8).

PnTXs

Oral LD_{50} values are only available for PnTX E+F, being 23 µg/kg b.w. following gavage and 60 µg/kg b.w. in food (addition to cream cheese) (Table 8). These values were estimated from the LD_{50} of algal extract containing approximately 10 µg PnTX/mg (Rhodes et al., 2010) and are the lowest for any CIs.

9.3. Relative potency of analogues

The toxicity of the CIs appears to be mediated via similar modes of actions by blocking of AChR receptors. The interaction with the receptors may differ between the different groups of CIs as both reversible and irreversible binding have been observed. In the absence of data on combined exposures it is reasonable to anticipate additive toxicity by the different analogues within each group of CIs.

Only a limited number of CIs have been determined in shellfish in Europe, 13-desmethyl SPX C is the SPX-analogue most commonly reported in Europe, but also SPX C and 20-methyl SPX G have recently been detected in over half of the samples in Norway (see Chapter 5.2). Occurrence data of SPXs have been reported as the sum of SPXs, which is the sum of masses of different SPXs, using, in the absence of specific standards, the MS response factor for 13-desmethyl SPX C in the quantification of the other SPX-analogues. By *i.p.* administration 13-desmethyl SPX C, SPX C and 20-methyl SPX G are equally toxic, therefore the current practice of expressing the sum of SPXs by using a factor of 1 for each analogue is justified.

For the other groups of CI toxins lack of data on occurrence and toxicity precludes a discussion on the relative potencies of the various analogues.

10. Observations in humans

No reports of human illness due to SPXs, GYMs, PnTXs or PtTXs have been identified. Episodes of toxicity, involving non-specific symptoms such as gastric distress and tachycardia were recorded in individuals in Nova Scotia, Canada consuming shellfish during times when SPXs were known to be present, but these could not be definitively ascribed to SPXs and are not consistent with the signs of toxicity in mice (Richard et al., 2001). Munday et al. (2004) noted that anecdotal reports from New Zealand indicate that individuals consuming shellfish contaminated with GYMs (concentrations not reported) suffer no adverse effects. Similarly, there have been no reported illnesses associated with seafood consumption in Rangaunu Harbour, New Zealand, where PnTXs have been detected in oysters at concentrations up to 200 µg/kg shellfish. In August 2008, a telephone survey was conducted in this region, involving 22 consumers of oysters and other seafood. None noted any illness associated with seafood. However, results of monitoring for PnTXs during this period are not available (McCoubrey, 2009).

11. Hazard characterisation

There are no long term studies on the groups of CIs in experimental animals that would allow establishing a tolerable daily intake (TDI). The toxins of various groups of CIs are characterised by binding to and blocking of AChR receptors in the central- and peripheral nervous system including neuromuscular junctions. The acute toxic signs have a rapid onset, in particular following *i.p.* administration. With regard to oral toxicity the reported toxicity varies greatly depending on whether the toxin is administered by gavage or in feed and whether the animal is fasted. In general, gavage administration shows lower LD_{50} values for the various toxins. In humans, no quantitative data on toxicity exist. In view of the acute toxicity of CIs the CONTAM Panel considered that an acute reference dose (ARfD) should be established for the different groups of CIs, but due to the lack of

adequate quantitative data on acute oral toxicity (i.e. no-observed-adverse-effect levels (NOAELs)) this was not possible.

12. Risk characterisation

From Table 8 it can be seen that the lowest oral LD_{50} values for SPXs in mice were in the region of 50 µg/kg b.w. and 500 µg/kg b.w. when SPXs were administered by gavage in fasted mice or in the feed, respectively. The CONTAM Panel concluded that these values did not provide an appropriate basis for establishing an ARfD, but calculated a margin of exposure (MOE) comparing the estimated dietary exposure in humans with these LD_{50} values. From the submitted data on SPX occurrence in shellfish, the 95th percentile of exposure, estimated using both deterministic and probabilistic approaches, is in the region of 0.06 µg/kg b.w. The MOE between this estimated high level of exposure and the LD_{50} values is in the range of 1000-10000. The lower end of this MOE range was based on the LD_{50} by gavage in fasted mice. The higher end of the range was based on the LD_{50} following administration of SPXs in the feed and is therefore more likely to be of relevance for the assessment of the risk of consumption of shellfish contaminated with SPXs. Taking also into account the steep dose response relationship observed in mice, as indicated by the small difference between the LD_0 and LD_{50} and the narrow confidence intervals of the LD_{50} values, and that mice given sublethal doses of SPXs are reported to make a rapid and full recovery, the CONTAM Panel concluded that current estimated exposure to SPXs does not raise concern for the health of the consumer. This conclusion is consistent with the anecdotal evidence of lack of ill effects in individuals eating shellfish containing CIs. However, the CONTAM Panel stresses that this conclusion for SPXs is based on very limited toxicity data.

Based on the occurrence data officially submitted to EFSA, exposure to other groups of CIs (GYMs, PnTXs and PtTXs) could not be estimated. Therefore no conclusions can be drawn with respect to any possible risk to consumers for these groups of CIs.

13. Uncertainty

The few data on occurrence of CIs in shellfish do not allow exposure assessment for the European population, except for the SPXs. In addition, there are limited animal toxicity data, and there are no reports of human illness attributed to CIs. Therefore, the CONTAM Panel concluded that the overall uncertainty is large and a detailed consideration of the various potential sources of uncertainty is not meaningful.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

General

- Spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) are cyclic imines (CIs), and are a family of marine biotoxins present in shellfish.

- SPXs are produced by the dinoflagellate *Alexandrium ostenfeldii*, whereas GYMs are produced by the dinoflagellate *Karenia selliformis*. The organism producing PnTXs has not been identified yet but has been described as a peridinoid dinoflagellate. PtTXs have only been detected in shellfish and no producing organism has been identified.

- SPXs, GYMs, PnTXs and PtTXs, like other CIs, are macrocyclic compounds with imine and spiro-linked ether moieties.
- Due to similarities in chemical structure and toxicity in mice these different groups of toxins have been grouped together.
- No information has been reported linking these compound groups to poisoning events in humans.

Methods of analysis

- The mouse bioassay (MBA) has been traditionally used to detect CIs. For reasons of animal welfare and poor specificity the MBA is not considered an appropriate detection method for CIs.
- The receptor-based fluorescence polarisation method has been shown to be able to detect GYM A and 13-desmethyl SPX C at relevant levels in shellfish. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific detection of individual CIs and they would be of value for their quantification in shellfish, subject to further development.
- None of the current methods of analysis to determine CIs in shellfish has been formally validated in interlaboratory studies.

Occurrence/Exposure

- SPXs have been identified in a number of European countries bordering the Mediterranean Sea, Atlantic coast and the North Sea.
- Three European Union (EU) member states reported results for SPXs in 1801 samples of which 80 % were below the limit of detection/limit of quantification (LOD/LOQ).
- To date GYMs have not been reported in shellfish produced in Europe, but they have been found in products imported from outside of Europe.
- PnTXs have so far only been identified in Norway, other European countries have not conducted surveys to this end.
- Recent findings suggest that PtTXs are transformed from PnTXs in shellfish. PtTXs have not been reported in shellfish in Europe.
- As for the previous scientific opinions on marine biotoxins, the Panel on Contaminants in the Food Chain (CONTAM Panel) identified the figure of 400 g as an appropriate large portion size to be used in acute exposure assessments.
- Using a deterministic approach, consumption of a 400 g portion of shellfish containing the sum of SPXs at 8.9 µg sum of SPXs/kg shellfish meat, corresponding to the 95th percentile of the concentration, would result in a dietary exposure of 3.6 µg sum of SPXs (equivalent to 0.06 µg sum of SPXs/kg body weight (b.w.) for a 60 kg adult).

- Using a probabilistic approach, the exposure distribution has median, mean and 95th percentile values of 0.44, 0.92 and approximately 3.4 µg of sum of SPXs per portion, equivalent to 0.007, 0.015 and 0.06 µg sum of SPXs/kg b.w, respectively for a 60 kg adult.

- There is no information available on the influence of processing on CI levels in shellfish.

Hazard identification and characterisation

- The acute toxicity of CIs, i.e. SPXs, GYMs, PnTXs and PtTXs, in rodents is characterised by the rapid onset of systemic neurotoxicity, including respiratory paralysis, and death within minutes, particularly following intraperitoneal (*i.p.*) administration. The toxic signs are consistent with the ability of these compounds to bind and block acetylcholine receptors in the central- and peripheral nervous systems, including neuromuscular junctions.

- Reported oral toxicity is usually lower than after *i.p.* administration. Gavage administration shows lower LD_{50} values than when the toxins are given in feed.

- The acute toxicity of 13-desmethyl SPX C, SPX C, and 20-methyl SPX G is similar, suggesting that the current practice of expressing the sum of these SPXs with a factor of 1 for each analogue is justified.

- No reports of human illness due to SPXs, GYMs, PnTXs or PtTXs have been identified. Anecdotal information suggests that illness has not occurred in regions in which CIs were detected in shellfish.

- There are no long term studies on CIs in experimental animals that would allow establishing a tolerable daily intake (TDI).

- In view of the acute toxicity of CIs the CONTAM Panel considered that an acute reference dose (ARfD) should be established for the different groups of CIs, but due to the lack of adequate quantitative data on acute oral toxicity this was not possible.

Risk characterisation

- The CONTAM Panel calculated a margin of exposure (MOE) between the lowest LD_{50} values for SPXs (in the region of 50 and 500 µg/kg b.w. when administered by gavage or in feed, respectively) and the estimated 95th percentile of exposure (0.06 µg/kg b.w.) from consumption of shellfish currently on the market. The MOE is in the range of 1000-10000.

- The lower end of this MOE range was based on the LD_{50} by gavage in fasted mice. The higher end of the range was based on the LD_{50} following administration of SPXs in the feed and is therefore more likely to be of relevance for the assessment of the risk of consumption of shellfish contaminated with SPXs.

- Taking also into account the steep dose response relationship observed in mice, and that mice given sublethal doses of SPXs are reported to make a rapid and full recovery, the CONTAM Panel concluded that current estimated exposure to SPXs does not raise concern for the health of the consumer.

- The CONTAM Panel stresses that this conclusion for SPXs is based on very limited toxicity data.

- Since exposure to other groups of CIs (GYMs, PnTXs and PtTXs) could not be estimated from the available data, no conclusions can be drawn with respect to any possible risk to consumers.

RECOMMENDATIONS

Methods of analysis

- Certified reference standards and reference materials for toxicologically relevant CIs need to be provided to allow method development, method validation and reliable application of analytical methodology in control programmes.

- Methods other than the MBA, in particular receptor-based fluorescence polarisation method for screening and LC/MS-MS methods for confirmation, should be further developed and optimised with respect to selectivity and sensitivity for CIs in shellfish. Subsequent (interlaboratory) validation studies are needed.

Occurrence/Exposure

- More information is needed on occurrence and stability of CIs in shellfish.

Hazard identification and characterisation

- Further information is needed to better characterise the oral toxicity of CIs and their relative potencies.

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ABBREVIATIONS

AChBP	Acetylcholine-binding receptor
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BTX	Brevetoxin
b.w.	Body weight
CCFFP	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
CI	Cyclic imines / confidence interval
CONTAM Panel	Panel on Contaminants in the Food chain
CRLMB	Community Reference Laboratory for Marine Biotoxins
CTX	Ciguatoxins
DA	Domoic acid
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
eq.	Equivalent
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental Oceanographic Commission of UNESCO/World Health Organization
GYM	Gymnodimines
HPLC	High-performance liquid chromatography
HPLC-UV	High-performance liquid chromatography-ultraviolet detection
i.c.	Intra-cerebroventricular
IOC	Intergovernmental Oceanographic Commission of UNESCO
i.p.	Intraperitoneal
ISO/IUPAC/AOAC	International Organization for Standardization/ International Union of Pure and Applied Chemistry/Association of Analytical Communities
JMPR	Joint FAO/WHO Meetings on Pesticide Residues
LB	Lower Bound
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
mAChR	Muscarinic acetyl choline receptor
MB	Median/Medium bound
MBA	Mouse bioassay
MLD	Minimum lethal dose
MOE	Margin of exposure
MS	Mass spectrometry

MU	Mouse Unit: the minimum amount needed to cause the death of an 18 to 22 g white mouse in 15 minutes
MW	Molecular weight
nAChR	Nicotinic acetyl choline receptor
ND	Not determined
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect level
NRCC	National Research Council Canada
OA	Okadaic acid
OJ	Official Journal of the European Union
P95	95th percentile
PlTX	Palytoxins
PnTX	Pinnatoxin
Post-MC	Post-market control
Pre-MC	Pre-market control
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
PtXS	Pteriatoxin
RBA	Rat bioassay
RSD	Relative standard deviation
SIM	Selected ion monitoring
SM	Shellfish meat
SPE	Solid Phase Extraction
SPX	Spirolide
SRM	Selected reaction monitoring
STX	Saxitoxin
TDI	Tolerable daily intake
UB	Upper Bound
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultraviolet
WG	Working group
WHO	World Health Organization
YTX	Yessotoxin